A META-ANALYSIS ASSESSING THE ROLE OF PHOSPHORYLATED TAU AND AMYLOID-BETA ON COGNITIVE DECLINE IN A PRECLINICAL MODEL OF ALZHEIMER'S DISEASE

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A META-ANALYSIS ASSESSING THE ROLE OF TAU AND AMYLOID-BETA ON COGNITIVE DECLINE IN A PRECLINICAL MODEL OF ALZHEIMER'S DISEASE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Aβ, amyloid-β	Amyloid-Beta
AD	Alzheimer's Disease
NFT	Neurofibrillary Tangles
GSK3	Glycogen Synthase Kinase-3
MWM	Morris Water Maze
NOR	Novel Object Recognition
RI	Recognition Index
3xTg-AD	Triple Transgenic Alzheimer's Disease Mouse Model
WT	Wild type mouse model
CPM	Counts per Minute

SUMMARY

Alzheimer's disease pathology is characterized by the accumulation of extracellular amyloid-beta (Aβ) plaques in the brain at onset followed by growth of phosphorylated tau proteins into neurofibrillary tangles (NFTs). Both Aβ plaques and NFTs interfere with normal neuronal cell function by disrupting proper synapse function at neural junctions and inflicting damage to neurons, eliciting cognitive decline. Based on previous literature, quantified accumulation of Aβ does not correlate with cognitive decline. There have been no FDA-approved drugs capable of curing AD or even delaying actual disease progression in patients with research efforts heavily focused on Aβ as the primary causative agent in AD pathology. Phosphorylated tau (pTau) accumulation into NFTs more strongly correlates with cognitive decline despite a smaller total concentration, and therefore, pTau may contribute more to Alzheimer's progression. We performed a meta-analysis combining and analyzing research studies matching inclusion criteria extracted from a database of more than 3,000 Alzheimer's mouse model peer-reviewed articles. Inclusion criteria encompasses: use of triple transgenic Alzheimer's Disease (3xTg-AD) mouse model, quantification of cognitive performance by Morris water maze (MWM) escape latency or Novel Object Recognition (NOR), and quantification of Aβ, total tau, and/or pTau levels. Phosphorylated tau, total tau, and Aβ levels were compared to measure the largest impact on cognitive performance by correlative assessment with MWM escape latency and NOR. Results indicate that increases in both pTau and Aβ levels in 3xTg-AD mice have significant correlation with and prediction of cognitive decline. Although pTau has a smaller concentration in the brain than Aβ, analysis of aggregated data illustrates that pTau has a stronger correlation

with cognitive decline. The two proteins interact through GSK3β and other phosphorylating pathways to cause more rapid cognitive decline, and the onset of Aβ may lead to the development of neurofibrillary tangles. Aβ and tau pathologies are interwoven in a complex manner that prevents clear separation. Alzheimer's disease treatment will require the removal and subsequent prevention of both proteins' accumulation. The present study concludes that future research should focus on combination drugs and defining the connection between Aβ and pTau rather than simply targeting Aβ inhibition alone.

CHAPTER 1

INTRODUCTION

Alzheimer's disease is the leading neurodegenerative disease in the world. Currently, 46 million people across the globe are afflicted with dementia (Alzheimer's accounts for around 70% of these cases), and the number is expected to grow to over 130 million by 2050 [1]. Over the course of 5-10 years, the disease slowly causes brain degeneration leading to dementia and eventually the inability to care for oneself. An estimated $818 billion is spent on dementia patient care each year including informal care provided and paid directly by family members [1]. The disease is costly to the patients financially, but moreover, it is emotionally taxing to their families who must look after and watch their loved ones suffer from debilitating cognitive deficits, including loss of functional memory, spatial coordination, and even self-identity. There is no definitive cure, and the only treatment simply delays the onset of symptoms without slowing disease progression [2, 3].

The hallmarks of Alzheimer's Disease (AD) pathology are marked by accumulation of extracellular amyloid-β (Aβ) plaques in the brain followed by intracellular neurofibrillary tangle (NFT) growth [4, 5]. Aβ upregulates the generation of NFTs by increasing glycogen synthase kinase-3 (GSK-3) activity, leading to the phosphorylation of tau (pTau) [6, 7]. Phosphorylated tau begins to self-assemble to form NFTs. Both Aβ plaques and NFTs interfere with normal neuronal cell function by disrupting synaptic signaling at neural junctions. Each protein's accumulation leads to neuron damage, eliciting diminished brain mass and cognitive function (Figure 1) [8]. Phosphorylated tau is linked to retarding Aβ growth establishing a negative feedback loop where increases in

pTau slows rate of Aβ accumulation [6]. As the disease progresses in late stages, pTau continues to form NFTs while Aβ plaque build-up plateaus. Connections between Aβ and pTau are not fully characterized, but current research is striving to completely describe the pathology of Alzheimer's disease.

The removal of Aβ plaques does not influence elimination of neurofibrillary tangles after NFTs have been established in the brain, but early intervention can prevent pTau development [9, 10]. Therefore, targeted late stage treatments may specifically eliminate Aβ without impacting pTau levels that have already accumulated. Comparison of differences in pTau and Aβ levels in treated mice illuminate differences between the proteins' impact on cognitive function. For example, phosphorylated tau levels were reduced by chemical treatment as Aβ levels continued to increase, yet cognitive function improved [11]. With decreasing pTau levels and increasing Aβ levels, cognitive improvement emphasizes that there is a quantitative difference between how the two proteins effect on cognitive deterioration. In the present study, relative level changes in both proteins were analyzed and correlated with the overall changes in cognitive ability to compare the impact. We also consider the interaction between tau and amyloid-β to understand why and how pTau levels decreased and Aβ increased.

Since the inception of the Amyloid Cascade Hypothesis, amyloid-β has been considered the main cause of degeneration in Alzheimer's, but there have been no definitive treatments or cures found to date [12]. The only FDA approved treatments are cholinesterase inhibitors, which increase acetylcholine levels to simply suppress the cognitive symptoms of Alzheimer's Disease, and memantine, which regulates glutamate to elicit neuroprotective effects but only mild cognitive improvement [2]. These drug

treatments simply mitigate the cognitive symptoms of AD without slowing disease progression [2, 3]. Treatments targeting Aβ and Aβ precursors have failed to pass clinical trials to improve patient outcomes [2, 3]. The presence of Aβ is associated with a decrease in cognitive performance; however, the level of Aβ inconsistently predicts the amount of cognitive decline [13]. Instead, it is suggested that other Alzheimer's pathologies, such as the hyperphosphorylation of tau, are the functional cause of degeneration after the initial onset of disease [8].

The present study compares the effects of Aβ and phosphorylated tau levels on cognitive performance through a large-scale meta-analysis of 3xTg-AD mouse model experiments. The triple-transgenic mouse model (3xTg-AD) of Alzheimer's expresses tangle and plaque pathology as well as synaptic dysfunction [14]. Aβ deposits first appear by 6 months of age while the first detection of phosphorylated tau occurs around 12 months of age [14, 15]. The 3xTg-AD mouse model pathology resembles human patients and has similar total tau levels in the cerebrospinal fluid [15-17]. The Morris water maze (MWM) reliably reflects how degradation of the hippocampus, striatum, basal forebrain, cerebellum, and neocortical areas affect cognitive function [18]. Meta-analysis was focused on correlations between cognitive performance in the Morris water maze or Novel Object Recognition test and amyloid-β and phosphorylated tau levels. To analyze the different Aβ forms and pTau epitopes, specific analyses were conducted (e.g. cognitive performance vs. soluble Aβ 42 and AT8 pTau antibody marker).

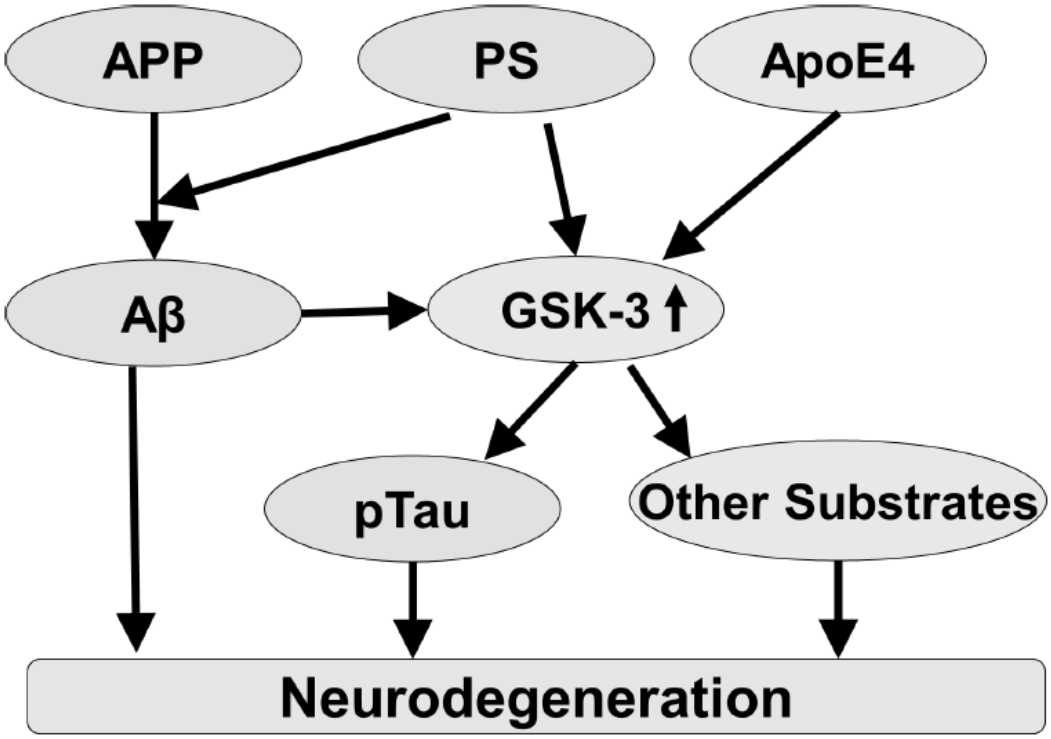


Figure 1. Pathology flowchart explaining connections between amyloid-β and tau phosphorylation. Even a simplified flowchart emphasizes the complexity and unknowns of the disease [6].

CHAPTER 2

METHODOLOGY

Systematic Review Process

We perform a systematic review, which quantitatively analyzes studies in a consistent manner to provide an over-arching view of a field's aggregated work. First, a series of database searches identified peer-reviewed publications that are likely to meet the study's criteria (Figure 2). These publications are then examined individually to determine whether they meet the inclusion criteria (see below). All quantifiable experimental data from figures and tables are extracted into an organized database in a process overseen by stringent quality control teams, resulting in transcription accuracy greater than 98.8% [19]. Comparable data are compiled to assess overall trends.

Inclusion Criteria

Key terms searches in PubMed were used to identify papers that were likely to meet the inclusion criteria. Search terms included "Alzheimer's Disease", "3xTg-AD", "amyloid-beta", "tau", "phosphorylated tau", "glycogen synthase kinase 3", and numerous combinations, variations, and abbreviations of these terms. For inclusion, peer-reviewed scientific studies must contain quantifiable data for either phosphorylated tau or Aβ levels and data for either Morris water maze escape latency or novel object recognition. Only papers utilizing the 3xTg-AD treated and control groups and published in English were included. Resultant searches led to the construction of a transcribed database of 3000 peer-reviewed articles that were assessed for inclusion. A total of 52 peer-reviewed journal articles met the required criteria for final inclusion.

3xTg-AD Mouse Model

The 3xTg-AD mouse model is a complex model with transgenic β-amyloid precursor protein (βAPPSwe), presenilin-1 (PS1M146V), and tauP301L gene replacements [15]. As in human Alzheimer's Disease pathology, both Aβ and pTau formation show age- and region-dependence with Aβ plaques forming before the accumulation of neurofibrillary tangles [20]. 3xTg-AD mice have phosphorylated human tau concentrations closer to human levels than P301L and Tg4510 transgenic AD mouse models [16]. Amyloid-β, total tau, and phosphorylated tau measurements were used from the mouse hippocampus for consistency and to assess the most direct impact on the morphological site of learning ability. Functional cognitive deficits appear as early as 4 months of age in the 3xTg-AD mice, a timeline that aligns with the formation of intracellular deposits of amyloid-beta in the hippocampus [21]. Both Aβ and pTau accumulate in significant levels in the brain after 8-12 months to contribute to cognitive deficits. The complex pathology in 3xTg-AD mice enables quantitative comparisons of Aβ and NFTs, as well as the ability to investigate possible interactions between Aβ and phosphorylated tau pathology.

Morris Water Maze

The Morris water maze (MWM), which tests spatial memory, was used as the primary measure of cognitive performance. The MWM is the de facto industry standard for cognitive tests for mouse models and was chosen because of its prevalence in AD experimental studies. A mouse is placed into a pool of water and trained to search for a visible platform in an open, cold (13°C) bath [22, 23]. After one day of training with the platform visible above water, the mouse is again placed in the maze with the platform

hidden below the water and uses visual cues outside of the bath to find the platform location. Training with the hidden platform continues four times a day for five to seven days. The time it takes for the mouse to find the platform is measured as the mouse's escape latency [24]. Escape latency data from day five was analyzed because it most highlights differences in learning within the standard practice of five days of training. Moreover, overtraining (too many trials per day or too many days of training) can result in all mice performing at a disproportionately high level and thus, preventing discernable quantitative learning differences [25]. The first two trials on day one serve as a control between mice for swimming performance and ability; mouse groups should not significantly differ on day one escape latency [23].

Novel Object Recognition

Due to the perceived limitations of the Morris water maze (e.g. mice are less suited to the task than rats, the stress caused by cold water may affect the results, it only tests spatial memory, etc.), novel object recognition (NOR) was used as a second measure of cognitive performance [26, 27]. NOR introduces mice to a new object and a familiar object to which the mouse had previously been exposed. Memory is then tested by the relative amount of time spent exploring the new object based on remembering the familiar object [28]. We chose recognition index (time spent exploring the novel object/ total time exploring) as the standard measurement for NOR. In cases where only percent time spent with novel object or discrimination index was provided, recognition index was calculated. Novel object recognition avoids stress on the mice and other factors such as swimming capability inherent to the Morris water maze. However, the disadvantage of

the NOR compared to the MWM is the inability to differentiate between the spatial and non-spatial conditions of the task [29].

Data Normalization

All data was normalized against untreated control 3xTg-AD mice within the same study to achieve a consistent baseline. Phosphorylated tau and amyloid-β were rarely measured in wild type (WT) mice, so WT mice could not be used as the primary control. Moreover, phosphorylated tau is usually measured in arbitrary units. A percent control calculation expresses a normalized data value: (Day 5 Escape Latency of treated 3xTg-AD experiment group) / (Day 5 Escape Latency of untreated 3xTg-AD control group) X 100%. Protein level can substitute for escape latency in the example above to attain equivalent percent control values for amyloid-β and pTau. There were no significant differences in variance (F-test $p = 0.8453$) or mean (t-test $p = 0.5008$) between MWM data normalized to day one compared to the non-normalized counterpart. Day 1 MWM training on a visible platform is consistent for all mice, so escape latency data was not normalized to day 1 to minimize data processing and alteration. When corresponding data was available, the same normalization was applied to wild type mice to assess relative protein levels and escape latency performance compared to untreated transgenic mice.

Statistical Analysis

We examined the correlation of escape latency or recognition index against tau, phosphorylated tau, and Aβ levels by performing a Pearson coefficient correlation analysis at alpha level 0.05. After determining significance, Pearson coefficient and slope allow comparison of the strength of the correlation. The Igor analytical software (Wavemetrics, Inc.) was used to calculate the line of best fit and the corresponding R^2

values. A principle component analysis was used to evaluate variance among kinases involved in the phosphorylation of tau; results were generated utilizing the built-in PCA function in MATLAB (The MathWorks, Inc.). A multiple linear regression also directly compared pTau and Aβ in studies that analyzed both protein levels; the MATLAB stepwise function established correlation factors between cognitive performance and protein variables at $\alpha = 0.05$ for significance. Clear outliers (9 data points) were eliminated by the Grubb test; graphical depiction and statistical analysis presented in the Results are without outliers.

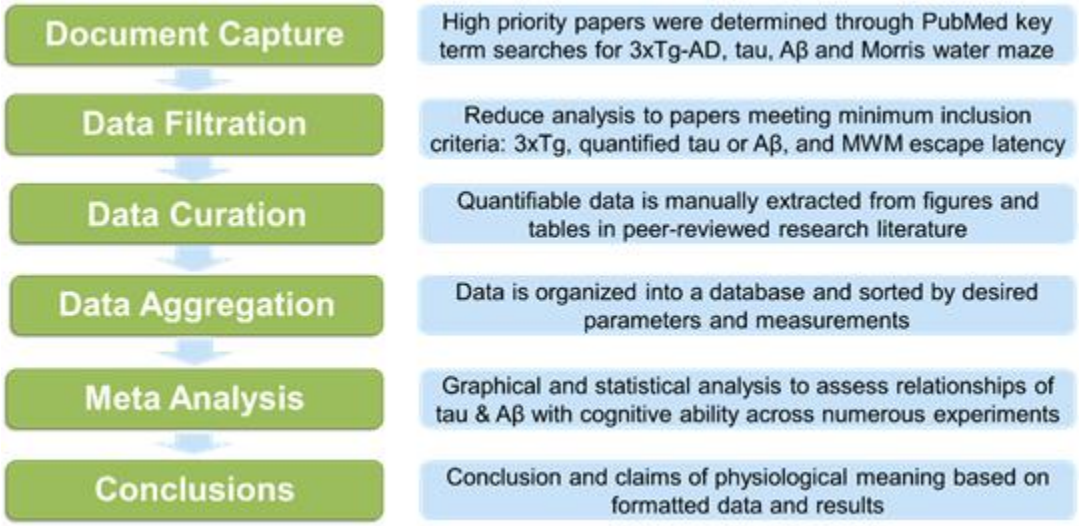


Figure 2. Flowchart detailing the process required for identifying and collecting data for meta-analysis.

CHAPTER 3

RESULTS

Amyloid- β Effects on Cognitive Decline

A comparison between Aβ, total tau, pTau, and cognitive function for 3xTg-AD mice contained over 300 mice from over 50 peer-reviewed scientific papers [17, 30-80]. Correlation of the relationship between total Aβ plaque levels and day five Morris water maze escape latency was measured by meta-data analysis (Figure 3A). Data points represent the aggregated average of a group of mice from a single experiment, where the line of best fit is weighted based on sample size for each aggregate point. Data point weighting and hippocampal protein measurements was consistent across all correlation analysis within our study. The Grubb outlier test identified 9 definitive aggregate data points as outliers, which were ultimately removed to ensure integrity of the analysis. We plotted the experimentally treated mouse escape latency normalized to a 3xTg-AD control mouse against the normalized total Aβ levels (Figure 3A). Total Aβ had a significant correlation to MWM escape latency ($p = 6.2768e-11$), but it was a very weak trend ($R^2 = 0.148971$). To support results in the Morris water maze, an equivalent test and set up was run for Novel Object Recognition (Figure 3B). NOR recognition index scores agreed with the results in MWM with a significant but very weak trend ($p = 0.0137$, $R^2 = 0.06369$).

To compare levels of each Aβ form (soluble Aβ42, soluble Aβ40, insoluble Aβ42, and insoluble Aβ40), individual analysis on the relationship between cognitive function and levels of each Aβ was completed. Percent control escape latency in the MWM at day five was plotted against each individual percent control in Aβ (Figure 4). Both soluble

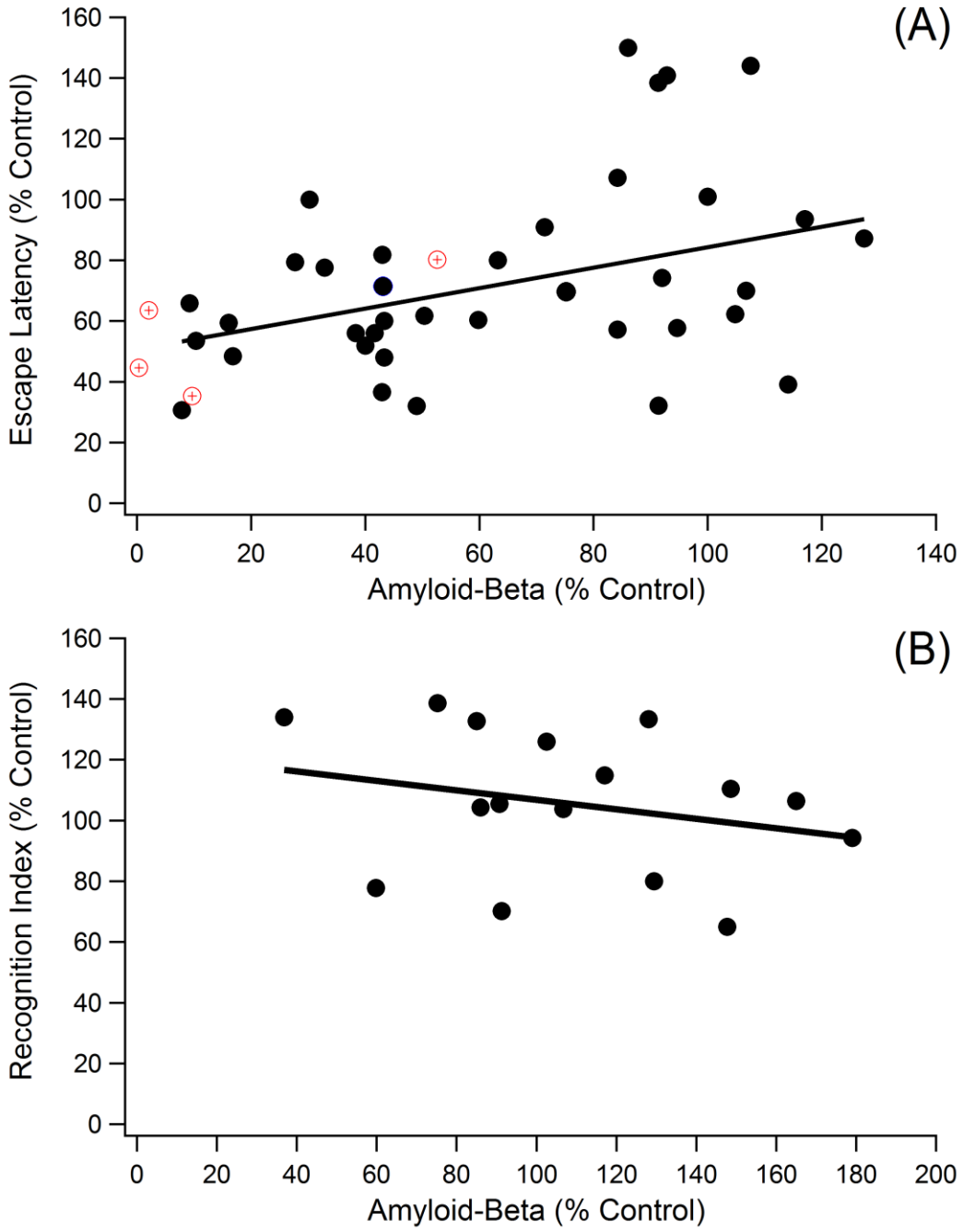
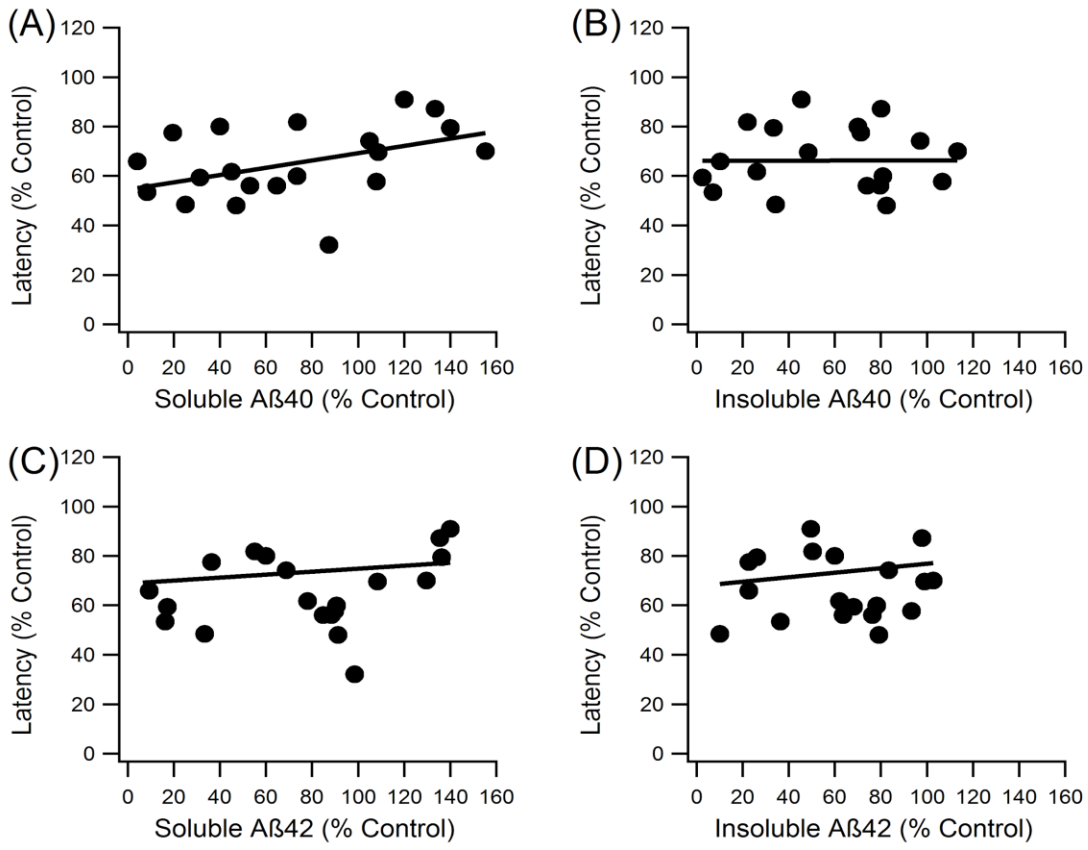


Figure 3. Increases in amyloid-β plaque level weakly correlates with decline in performance in both the Morris water maze and the Novel Object Recognition test. A) A positive trend indicates that as Aβ increases, cognitive performance declines (slope = 0.33614, R^2 = 0.148971, n = 259). The red target markers provide a visual reference for wild type mouse measurements in comparison to 3xTg-AD control mice. B) Novel object recognition correlation results in similar significant, weak trends, where a negative trend indicates decreased cognitive function with Aβ increases (slope = -0.15599, R^2 = 0.06369, n = 116).

Aβ forms showed weak, but significant trends with cognitive function (n = 148, p = 6.4058e-11, slope = 0.14792, R^2 = 0.247293; p = 1.2842e-07, slope = 0.13503, R^2 = 0.1667, for Aβ40 and Aβ42, respectively) (Figure 4A & 4C). Insoluble Aβ40 and Aβ42 showed no significant correlation with MWM escape latency (Figure 4B & 4D). Both trends had nearly flat lines with very low R^2 values indicating that insoluble Aβ levels did not correlate with cognitive function (R^2 < 0.00125, slopes < 0.005, p > 0.44, n = 144). Amyloid-β data measurements fell into two main categories: direct (staining and imaging of plaques or western blot with total Aβ marker like 6E10) and indirect (calculated combination of Aβ individual forms). The direct measurements had a significant correlation with escape latency (p = 2.8755e-14) (Figure 2E).



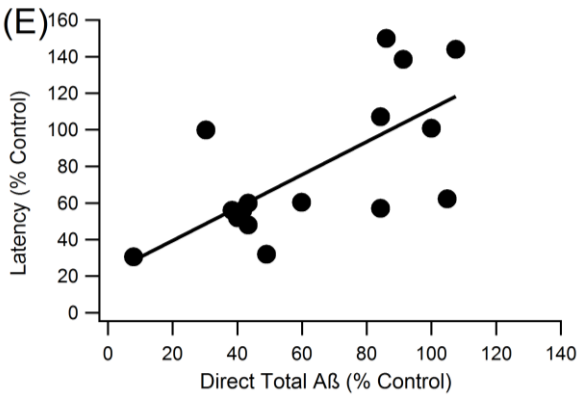


Figure 4. Correlation analysis of each major amyloid-β form to Morris water maze escape latency. A, C) The soluble forms of Aβ showed significant correlation with escape latency performance, and the slope of the trend was similar to total Aβ (n = 148; Aβ40: slope = 0.14792, R^2 = 0.24729; slope = 0.13503, R^2 = 0.1667. B, D) Neither Insoluble Aβ40 nor Aβ42 displayed significant trends with cognitive function (p = 0.4865, p = 0.4470, respectively). E) Direct Aβ had a significant trend with cognitive function (slope = 0.9009, R^2=0.4640, n = 93).

Changes in Total Tau Levels Has No Impact on Cognitive Decline

After confirming previous results that Aβ and its subforms only weakly correlates with cognitive function in the Tg2576 model, total tau and phosphorylated tau were analyzed for their contributions to Alzheimer's pathology [13]. Total Tau (unphosphoryated + phosphorylated tau) showed no significant trend in either Morris water maze or Novel Object Recognition (Figure 5). Total tau, also referred to as tau in the present study, is measured by anti-human tau antibody HT7 or Tau-5 which bind to location on tau that allow recognition of both unphosphorylated and phosphorylated tau [81, 82]. In fact, the trend slopes even indicate increases in total tau improve cognitive function. Most data points were centralized around 100% of control for total tau indicating total tau levels in treated mice did not change much compared to the control. Pearson coefficient analysis for escape latency versus total tau produced a p value of 4.5447e-04 and R^2 = 0.0709 (n = 152). Novel object recognition had a nearly flat trend line with most tau data again clustered around 100% of control (p = 0.2510, R^2 = 0.005517, slope = 0.04653, n = 85).

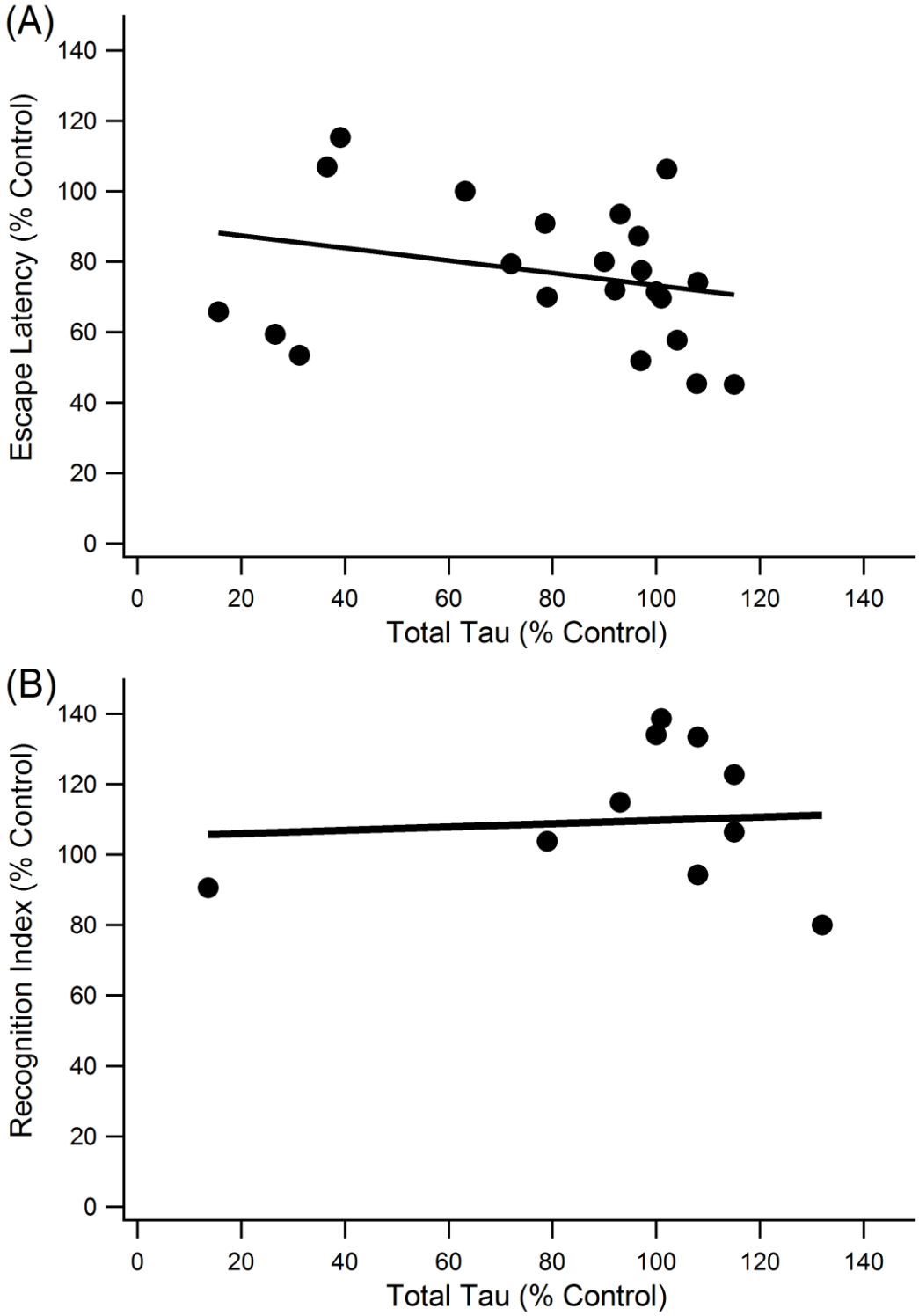


Figure 5. Total tau does not significantly correlate with cognitive decline measured by the Morris Water Maze or Novel Object Recognition. A) There was a weak, significant trend for day five escape latency with total tau levels ($p = 4.5447e\text{-}04$, fit = $90.936 – 0.17679x$, $R^2 = 0.0709$, $n = 152$). B) Tau levels did not correlate significantly with performance in the Novel Object Recognition test ($p = 0.2510$).

Phosphorylated Tau Impacts Cognitive Decline

Although analysis of total tau showed no association with cognitive ability, phosphorylated tau has a significant association with cognitive ability through both Morris water maze and Novel object recognition correlations (Figure 6). Phosphorylated tau was measured through western blot, imaging of the hippocampus, or ELISA. In all cases, expression units were presented as relative to a 3xTg-AD control. Imaging of immunohistologically marked phosphorylated tau provides direct measurement of neurofibrillary tangles. The other measurements also indicate hyperphosphorylation and accumulation of NFTs.

Phosphorylated tau correlation with Morris water maze escape latency surpassed amyloid-β with a steeper slope of 0.40837 and $R^2 = 0.2753$. To confirm the comparison between pTau and Aβ, the proteins were used as variables to predict escape latency performance in a multiple linear regression. Using the MATLAB stepwise function on data points where pTau and Aβ were available, it was determined that pTau was a stronger variable input for predicting cognitive function than Aβ. Both variables considered alone were significant factors for improving correlation, but adding Aβ variable to the pTau trendline did not significantly improve correlation (data not shown).

Phosphorylated tau epitopes are expressed inconsistently between papers as well as within the same paper. There is not a definitive epitope that is considered the most impactful or completely irrelevant. AT8, AT270, AT180, and PHF-1 monoclonal antibody markers were compared to determine if one had a more consistent measurement and correlation with MWM performance (Figure 7). Both AT8 and AT270 were significant and had relatively strong, positive correlation coefficients ($r = 0.7217$ and $r =$

0.8280). PHF-1 and AT180 had no significant trends with escape latency, and they even have negative slopes (r = -0.0646 and r = -0.4922).

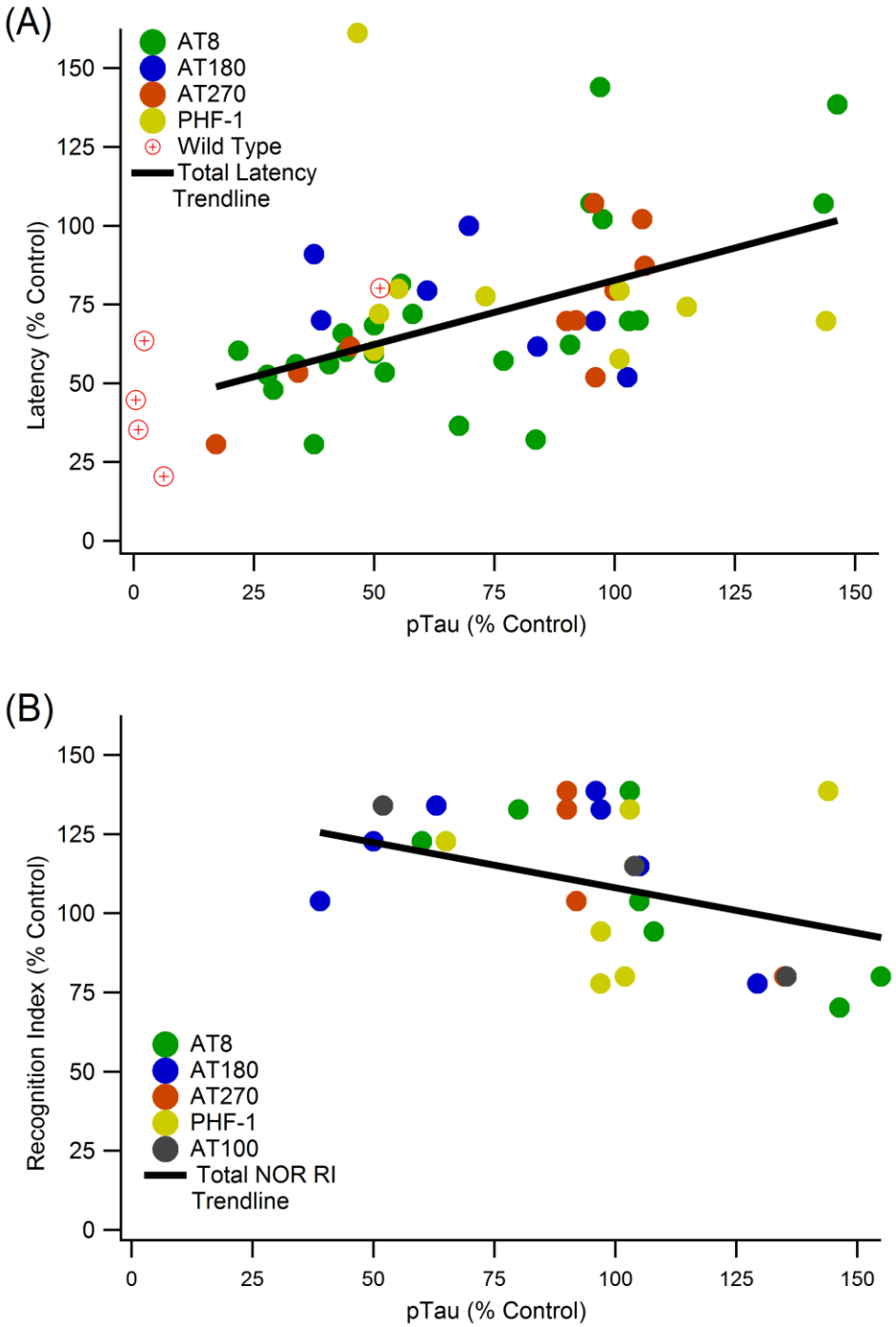


Figure 6. Phosphorylated Tau correlates with both cognitive function tests. A) A significant positive slope with escape latency indicates cognitive function decreases as pTau levels increase ($p < 1.0e-15$, fit = 41.85+ 0.40837x, $R^2 = 0.2753$, n = 371). B) A significant negative slope with Novel Object Recognition indicates cognitive function decreases as pTau levels increase ($p = 0.0117$, fit = 14.294 – 0.31962x, $R^2 = 0.1763$, n = 113).

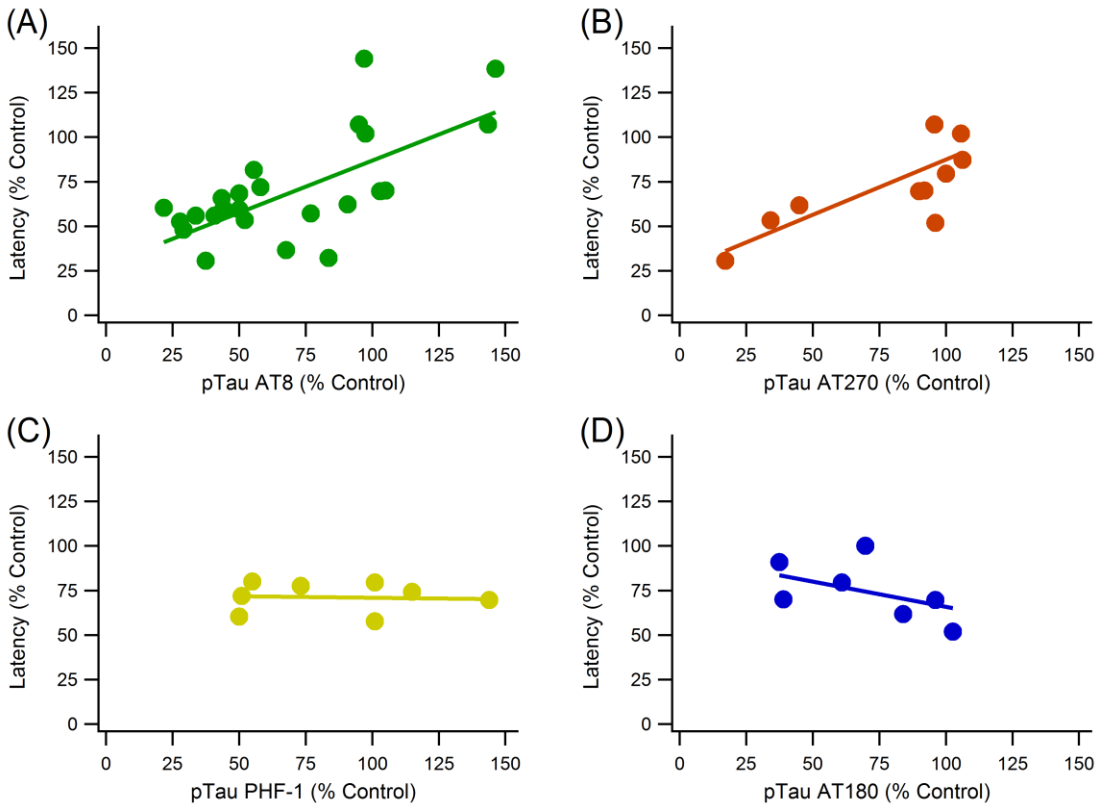


Figure 7. Phosphorylated tau epitope analysis comparing different phosphorylation locations. Phosphorylated Tau epitope breakdown shows strong positive correlations with AT8 (A) and AT270 (B) while showing no trend or a weak, negative trend with PHF-1(C) and AT180 (D). A) AT8: fit = 28.3 + 0.58555x, $p < 1.0e\text{-}15$, $R^2 = 0.5209$, n = 185. B) AT270: fit = 25.39 + 0.61933x, $p < 1.0e\text{-}15$, $R^2 = 0.685582$, n = 88. C) PHF-1: Fit = 72.638 - 0.01739x, p = 0.3295, $R^2 = 0.004179$, n = 48. D) fit = 94.28 - 0.2854x, p = 3.4486e-04, $R^2 = 0.2423$, n = 44. Sample sizes noticeably decreased from A to D due to a lack of data and measurements using the AT180 and PHF-1 markers.

Principal Component Analysis

A principal component analysis (PCA) calculated contribution of individual components to variance in the data and provided an overall assessment of disease complexity. Additionally, kinases involved in tau phosphorylation (pGSK3β, GSK3β, p25, and p35) were analyzed to identify possible targets for future research (Figure 8). PCA calculates components that explain the largest amount of variance, whereas a biplot displays how much variables contribute to variance for a particular component. Six outlier data points were removed from Figure 8A-C for the analysis in 8D-F; although

appearing different at first, the important considerations are orthogonality of the variables and overall contribution to variance explained. Correlating variables cluster together on the biplot and contribute similarly to variance of the pathological "system". Phosphorylated GSK3β contributed most strongly to variance in the "system", and pTau and MWM escape latency contribute to variance in a collinear manner.

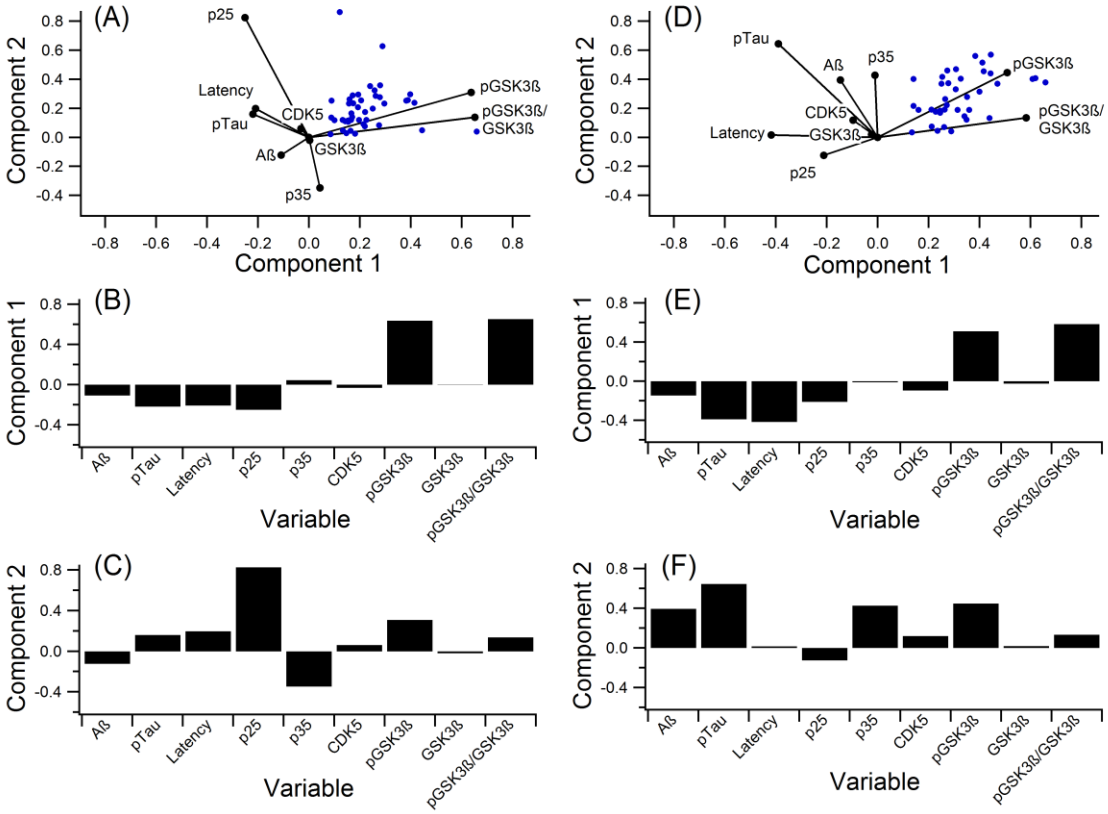


Figure 8. Principal component analysis to determine variance contribution of the key components in tau phosphorylation. (A-C) PCA on all data with no outlier removal graphed in a biplot (A) and broken down to clearly show the first two components (B, C). Component 1 accounted for 63.6% of the variance, and component 2 accounted for 27.9%. (D-F) PCA on data with six outliers removed displayed in a biplot (A) and broken down to show the first two components (E, F). Component 1 accounted for 86.6% of the variance, and component 2 accounted for 13.4%.

CHAPTER 4

DISCUSSION

Total direct Aβ and phosphorylated tau both correlated with cognitive decline in the Morris water maze and Novel object recognition. However, phosphorylated tau had a stronger and more definitive trendline, indicating that with a lower overall concentration, pTau has a stronger connection to cognitive ability in transgenic mice than does Aβ [83]. Individual Aβ form analysis supports previous findings that Aβ correlates weakly with cognitive decline and has a limited effect on cognitive changes [13]. Inconsistent trends amongst the pTau antibodies presents an interesting discussion on the quality of pTau epitope markers and phosphorylation location influence on self-assembly into neurofibrillary tangles. Principal component analysis emphasized the need for further investigation of tau phosphorylation mechanisms and the intricate complexity of Alzheimer's Disease pathology, which suggests the need for combinatorial therapeutic strategies.

Pathology Changes in Wild Type and 3xTg-AD Mice Lead to Corresponding Cognitive Outcomes

Relative pTau and Aβ protein levels of wild type mice to 3xTg-AD control mice coincided with relative escape latencies of treated 3xTg-AD mice with a similar reduction in pTau and Aβ. A 50% decrease in pTau led to a 30-50% reduction in escape latency for both treated 3xTg-AD and wild type mice. The alignment of wild type data supports the validity and consistency of using 3xTg-AD control mice. Moreover, wild type mice provide the lower limit on how fast the mice can swim and find the platform with no Alzheimer's Disease pathology [25].

Amyloid-β Displays Weak Trends with Cognitive Ability

Total amyloid-β showed only weak trends with cognitive decline, which was supported by the individual analysis of the four main forms of the Aβ components. When total amyloid-β was not measured, the four Aβ forms presented in this study were added together for a total Aβ calculation. As expected, the trends with insoluble Aβ were insignificant and not an indication of cognitive decline. Despite insoluble Aβ typically having higher concentrations in the brain, relative changes in soluble Aβ levels had a larger impact on cognitive function [36, 57, 76]. The diffuse nature of soluble Aβ allows it to have a more direct impact on neurons by surrounding the cell surface and synapses leading to neurotoxicity [84-86]. The connection between Aβ and tau phosphorylation is not well defined, but significant trends could indicate soluble Aβ oligomers have a stronger influence in activating kinases for tau phosphorylation [87]. Direct Aβ measurements correlated strongly with escape latency ($R^2 = 0.769$, $r = 0.8768$) (Figure 2E), but pTau correlated even more strongly with escape latency for points where pTau measurements were available (9 out of 12 data points available; $R^2 = 0.847$, $r = 0.9204$). These trends support the indirect role that amyloid-β plays in the Amyloid Cascade Hypothesis, but the variance and weak trendlines reinforce the complexity of the interaction. Thus, while amyloid-β may be the most physically visible marker of AD, it's actual role in the disease etiology, and namely, cognitive decline, is neither independent nor direct, but rather, intertwined with mechanisms like pTau and GSK-3.

Total Tau and Phosphorylated Tau Pathology

Total tau did not have a clear impact on cognitive decline, and trends even indicated that it may improve cognitive ability. The result is unsurprising considering tau is a normal and prevalent protein in the brain that is needed for microtubule stabilization

and proper neuron function [88, 89]. Additionally, tau does not aggregate into NFTs even when overexpressed because of its natural high solubility [90]. Trends were unclear because tau in treated mice did not differ from the control mice substantially in many experiments; the measurements tended to be within 10-20% of the control value. Such a sensitive range may not allow meaningful assessment of cognitive impact with MWM and/or NOR, especially in a complex disease with numerous other contributing factors.

Phosphorylated tau, however, showed strong trends with cognitive performance in both cognitive tests. Individual breakdown of pTau epitopes (AT8, AT180, AT270, and PHF-1) revealed there were differences in predicting cognitive function. AT8 and AT270 had a strong correlation between increasing levels and cognitive decline; however, AT8 had more supporting data that promoted a strong correlation between increased pTau levels and increased latency in the Morris water maze. Therefore, AT8 could be the most effective and consistent method in measuring phosphorylated tau level. Both PHF-1 and AT180 resulted in very low correlation between escape latency and pTau levels, but both trends could have been impaired by insufficient sample size. Further research on the binding characteristics is needed to understand accuracy of measurement and to consistently explain differences in measurements. The presented results of our aggregated analysis beg the question, "Is there a lack of consistency between the experimental antibodies, or does phosphorylation location impact the ability to self-assemble into neurofibrillary tangles?"

Comparing Phosphorylated Tau and Amyloid-Beta

Phosphorylated tau has a significantly more powerful impact on cognitive function than amyloid-β, especially considering pTau deposits are not as substantial, in

terms of visual prevalence and concentration in cerebrospinal fluid in the brain [83, 91]. Phosphorylated tau is most likely the primary factor in cognitive decline, though Aβ may play an indirect role in Alzheimer's pathology for development of neurofibrillary tangles. Based on the 3xTg-AD mouse model, Aβ is the first developing pathology in Alzheimer's disease, suggesting that it may serve as the first link in the chain leading to neurodegeneration by influencing tau phosphorylation. Our conclusions support findings of previous studies; Foley et al. indicates that Aβ may cause some cognitive decline when highly concentrated, but shows that quantitative levels do not correlate with cognitive decline. In contrast, levels of pTau do significantly and directly correlate with cognitive decline, and therefore, pTau could be a key functional treatment target even in later stages of the AD pathology. The connection between phosphorylated tau and amyloid-β requires further research to define. It is likely, however, that Aβ indirectly promotes tau phosphorylation through upregulation of kinases such as GSK-3β and p25, which phosphorylate tau.

Principal Component Analysis Emphasizes Complexity of Alzheimer's Disease

Glycogen synthase kinase-3 (GSK-3), p25, p35 are kinases involved in the phosphorylation of tau as an important step in AD pathology [7, 92]. Analysis focused around GSK-3 due to its dominance as a tau phosphorylation kinase and prevalence in literature; specifically, the GSK-3β isoform is linked more closely with tau pathology [92]. In both analyses (Figure 8), the Morris water maze escape latency variance is low in both components; this finding is somewhat expected because there is a natural limit to the variance in escape latency. In both analyses, phosphorylated GSK3β (pGSK3β) and phosphorylated GSK3β normalized to total GSK3β (pGSK3β/GSK3β) contribute

substantially to variance, which highlights them as prime future research targets to assess mechanisms directly related to escape latency. Total GSK3β contributed little to variance as most of the values remained around 100% of a 3xTg-AD control mouse; therefore, the similar results of pGSK3β and pGSK3β/GSK3β is expected. Phosphorylated tau and latency align in the analysis with outliers and closely in component 1 in the no outliers analysis. These findings suggest the presence of collinearity and provide corresponding additional support that pTau more directly influences cognitive decline than Aβ in the 3xTg-AD pathology.

CHAPTER 5

CONCLUSION AND FUTURE WORKS

Conclusion

From the lack of drug development and the corresponding support of large-scale meta-analysis, Aβ may not be the most crucial therapeutic target to improve Alzheimer's Disease symptoms (Figure 9). Future research should focus on eliminating phosphorylated tau to reverse Alzheimer's disease symptoms, rather than focusing solely on Aβ elimination. Due to the complex interactions between multiple proteins, a multifaceted treatment approach is the best means by which to combat the disease. Furthermore, protein regulator treatments could moderate the activity of kinases like pGSK3β to prevent hyperphosphorylation of tau, yet allow normal pGSK3β function without complete elimination. The large variance in all aspects of the trend lines and moreover, the number of variance-contributing variables in principal component analysis, suggests that many other factors are affecting the overall outcome of cognitive ability in AD. A larger research focus on higher level interactions such as Aβ and GSK-3β with tau phosphorylation will give a better understanding of the disease etiology and more likely lead to a cure. Targeting both pTau and Aβ could decrease direct neuron disruption and may help prevent subsequent protein accumulation. The results of this systematic review and meta-analysis comparing Aβ and tau steer the field in a new direction toward phosphorylated tau and combination therapies to more effectively treat Alzheimer's Disease.

Future Works

The work presented in this thesis, which comprehensively examined the impact of phosphorylated tau and Aβ and their effect on cognitive decline in the 3xTg-AD mouse model, will be submitted for peer-review for possible research journal article publication in May 2017. While my future in Dr. Mitchell's lab will end upon my May 2017 graduation, new projects will expand off the present work to further assess and compare overall data trends in Alzheimer's disease. Future studies may include a more in depth view into the biochemistry of Aβ and tau pathology, or even a confirmation of results in another mouse model. Directions that would best assist the field for new research routes would include informatics analysis of other Alzheimer's Disease factors such as inflammation, oxidative stress, or specific connections of Aβ and pTau to further evaluate the Amyloid Cascade Hypothesis or to identify other promising hypotheses.

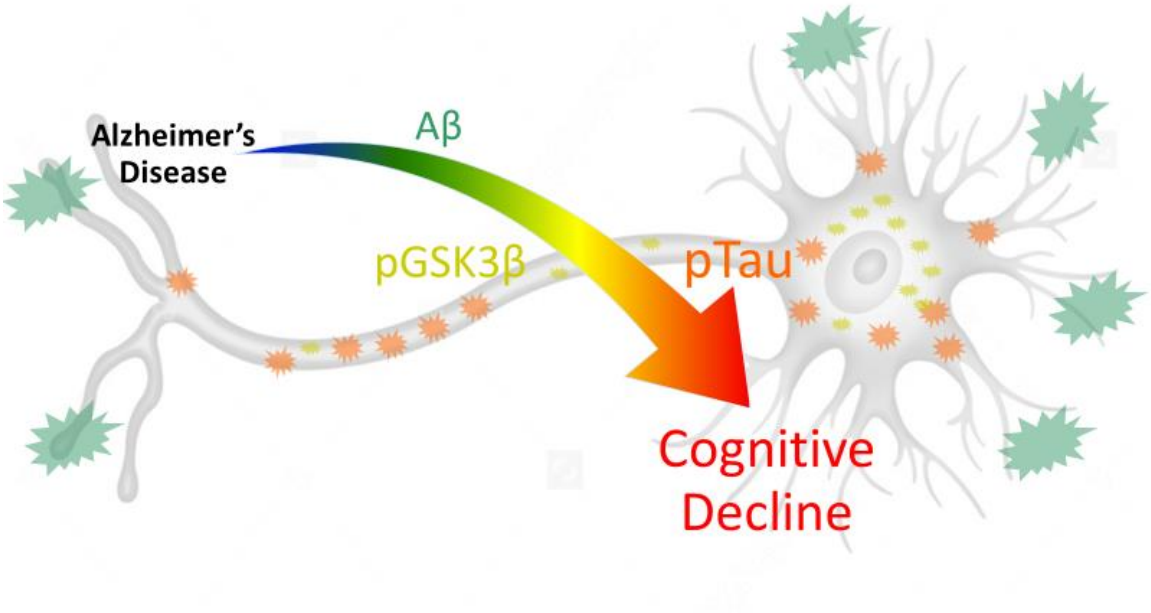


Figure 9. Summative figure emphasizing the contribution of pTau to cognitive decline and the demand for further research in understanding tau phosphorylation and the connection to Aβ.

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